EXHIBIT C

Antimutagenic Properties of 3,5-Disubstituted 2-Thiohydantoins

Asaka Takahashi,[†] Hiroki Matsuoka,[‡] Yoshio Ozawa,[‡] and Yasushi Uda*,[†]

Department of Bioproductive Sciences, Faculty of Agriculture, Utsunomiya University,
Utsunomiya 321-8505, Japan, and Gunma Women's Junior College, Takasaki 370-0033, Japan

3,5-Disubstituted 2-thiohydantoins, which were prepared by the reaction of allyl isothiocyanate (AITC) or 4-(methylthio)-3-butenyl isothiocyanate (MTBI) with various amino acids, were assayed for their inhibitory effects on the mutagenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) on *Salmonella typhimurium* TA 98. When the 3,5-disubstituted 2-thiohydantoins (125–500 μg/mL) were simultaneously preincubated with the bacterial strain, IQ (0.5 μg/mL), and rat microsomal fraction (S9 mix) for 20 min, a dose-dependent inhibition of IQ mutagenicity was observed in the tested compounds except one prepared from MTBI and L-methionine. This inhibition ranged from 23% to 86%. The inhibitory effect either disappeared or was largely reduced to 5–19%, when the 3,5-disubstituted 2-thiohydantoins were added after metabolic activation of IQ with S9 mix. These results suggest that the 3,5-disubstituted 2-thiohydantoins probably acted as an inhibitor of the S9 mix-mediated metabolic activation of the mutagen.

Keywords: *2-Thiohydantoins; allyl isothiocyanate; 4-(methylthio)-3-butenyl isothiocyanate; blocking agent; Salmonella typhimurium TA 98*

INTRODUCTION

A variety of isothiocyanates are generated in cruciferous vegetables (VanEtten and Wolff, 1973). Of these, allyl isothiocyanate (AITC) is used as a condiment or a food additive. Isothiocyanates, due to their electrophilic nature, react with compounds possessing a hydroxy, thiol, or amino group to form corresponding thiocarbamates, dithiocarbamates, and thioureas (Ashworth, 1972). *p*-Hydroxybenzyl isothiocyanate in white mustard paste is known to be easily degraded into dibenzyl sulfide and benzyl alcohol (Kawakishi et al., 1967). AITC is also unstable in an aqueous medium and degrades to form a variety of sulfur-containing products such as diallyl mono-, di-, tri-, and tetrasulfides, 1,2-dithiolene, and 1,2,3-trithiin (Kawakishi and Namiki, 1969; Chen and Ho, 1998). Radish pungent principle, 4-(methylthio)-(*E,Z*)-3-butenyl isothiocyanate (MTBI), has recently been reported to be highly reactive with water or alcohol to form thioxopyrrolidines such as 3-(hydroxymethylene)-2-thioxopyrrolidine, 3-[(methylthio)methylene]-2-thioxopyrrolidine, and methyl 4-(methylthio)-(*E,Z*)-3-butenyl dithiocarbamate (Kosemura et al., 1993; Matsuoka et al., 1997; Uda et al., 1990). As for the reaction of isothiocyanates with amino acids, the Edman degradation of proteins and/or peptides is well-known (Edman, 1950). The kinetics and mechanism of the formation of the 3,5-disubstituted 2-thiohydantoins were described by Drobnica and Augustin (1965a,b). It is thought that, during the cooking or processing of food and also in the human digestive system, isothiocyanates in foods react partly with free amino acids to form their corresponding 2-thiohydantoins. Therefore, it is important to obtain information about the physiological and biological properties of the

2-thiohydantoins that are formed from naturally occurring isothiocyanates and common amino acids in foods.

There have been many reports on the biological activities of 2-thiohydantoins. For example, they can act as antitumor (Al-Obaid et al., 1996), antiviral (El-Barbary et al., 1994), antibacterial (Froelich et al., 1954), antifungal (Marton et al., 1993), and anticonvulsant (Cortes et al., 1985) agents. Some hydantoins and 2-thio analogues can induce a lymphoproliferative popliteal lymph node reaction (Kammueller and Seinen, 1986; Marintchev et al., 1995). To the best of our knowledge, no information is available about antimutagenic and/or anticarcinogenic activities of 3,5-disubstituted 2-thiohydantoins that are derived from naturally occurring isothiocyanates and amino acids. It is known that a variety of mutagens and carcinogens are formed in foods during cooking and processing. In cooked foods such as fried or broiled meat and fish, several heterocyclic amines including 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) can be produced via amino acid pyrolyzation or by reaction of creatin(in)e with Maillard reaction products (Hatch et al., 1984; Jägerstad et al., 1983; Spingarn et al., 1980; Sugimura et al., 1977). IQ, one of the common food-born heterocyclic amines, is activated in mammalian liver by cytochrome P-450-dependent N-hydroxylation to form proximate mutagen (Abu-Shakra et al., 1986; Yamazoe et al., 1983) and is not only mutagenic in the Ames/*Salmonella* assay (Kasai et al., 1980) but also carcinogenic to give tumors at a number of sites in mice and rats (Ohgaki et al., 1984, 1991; Takayama et al., 1984). On the other hand, various types of compounds possessing an inhibitory activity against mutagens and carcinogens also occur in food systems. In cruciferous vegetables, glucosinolate-derived isothiocyanates and indoles have been reported to act as antimutagenic and/or anticarcinogenic agents. For example, benzyl, 2-phenylethyl (PEITC), and 4-methylsulfinylbutyl isothiocyanates have been shown in *Salmonella* (*S. typhimurium* strains TA 98,

* To whom correspondence should be addressed [fax, (81) 28-649-5401; e-mail, uda@cc.utsunomiya-u.ac.jp].
[†] Utsunomiya University.
[‡] Gunma Women's Junior College.

TA 100, and TA 1535) assays to act as antimutagens against heterocyclic amines including IQ via an inhibition of cytochrome P-450-mediated metabolic activation of the mutagens (Barcelo et al., 1996; Hamilton et al., 1994; Hamilton and Teel, 1996; Knasmuller et al., 1996). These isothiocyanates can induce phase II detoxifying enzymes, glutathione *S*-transferase, and quinone reductase in various mammal tissues (Zhang and Talalay, 1994). In fact, the isothiocyanates showed a clear suppressive effect on chemical carcinogen-induced mammary tumor models (Wattenberg, 1977, 1987; Morse et al., 1989, 1991) and, thus, are considered to be cancer-protective. Indole-3-carbinol, 3,3'-diindolylmethane, and indole-3-acetonitrile occurring in edible cruciferous vegetables have been shown to be inhibitors of either 7,12-dimethylbenz[*a*]anthracene-induced mammary tumor formation in rats or benzo[*a*]pyrene-induced neoplasia of the forestomach in mice (Wattenberg and Loub, 1978), though indole-3-acetonitrile was converted to a mutagenic 1-nitrosoindole-3-acetonitrile after nitrite treatment (Wakabayashi et al., 1985).

It is interesting to investigate whether 3,5-disubstituted 2-thiohydantoins derived from isothiocyanates and amino acids have an antimutagenic activity as well as isothiocyanates. The present paper deals with the antimutagenic properties of 3,5-disubstituted 2-thiohydantoins that have been prepared from AITC or MTBI and various amino acids against IQ on *S. typhimurium* TA 98. This report also describes the effect of pH on the formation of the 3,5-disubstituted 2-thiohydantoins and on the growth of some microorganisms.

EXPERIMENTAL PROCEDURES

Materials. A mixture of (*E*)- and (*Z*)-MTBI (about 7:1) was prepared from radish seedlings by our previously described method (Uda et al., 1990). AITC and IQ (*caution:* carcinogen) were purchased from Wako Pure Chemical Inc. (Tokyo, Japan) and used without further purification. *S. typhimurium* TA 98 was provided by the National Institute of Health Sciences (Tokyo, Japan). S9 fraction and cofactors composed of magnesium chloride hexahydrate (80 μmol), potassium chloride (330 μmol), glucose-6-phosphate (50 μmol), nicotinamide adenine dinucleotide phosphate reduced form (40 μmol), nicotinamide adenine dinucleotide reduced form (40 μmol), disodium hydrogen phosphate (842 μmol), and sodium dihydrogen phosphate dihydrate (158 μmol) were obtained from Oriental Yeast Co. (Tokyo, Japan). The cofactors dissolved in 9.0 mL of distilled water were filtrated with a sterilized membrane filter (0.22 μm) and mixed with 1.0 mL of the S9 fraction. This was immediately used as S9 mix.

Instrumental Analysis. Ultraviolet (UV) spectra were obtained in ethanol (EtOH) using a Hitachi 330 double-beam spectrophotometer. Infrared (IR) spectra were recorded in potassium bromide (KBr) on a Horiba 1200 FT-IR spectrometer. High-resolution (HR) and low-resolution (LR) electron impact mass spectral (EI-MS) analysis was done on a JEOL AX-500 mass spectrometer at 70 eV. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured in acetone-d_6 ((CD$_3$)$_2$CO) or chloroform-d (CDCl$_3$) on a JEOL EX-400 nuclear magnetic resonance spectrometer using tetramethyl-silane (TMS) as the internal standard.

Preparation and Structural Confirmation of 3,5-Disubstituted 2-Thiohydantoins. A mixture of MTBI (containing in total of 1.0 mmol of the isomers) or AITC (1.0 mmol) and the equivalent amount of amino acid (L-valine (Val), L-leucine (Leu), L-phenylalanine (Phe), L-methionine (Met), and L-tryptophan (Trp)) in 50 mL of MacIlvaine (citric acid–disodium hydrogen phosphate) buffer (pH 8.0) was stirred for 48 h at 86 °C. The mixture was extracted with ethyl acetate. The extract, after drying on a powder of anhydrous sodium
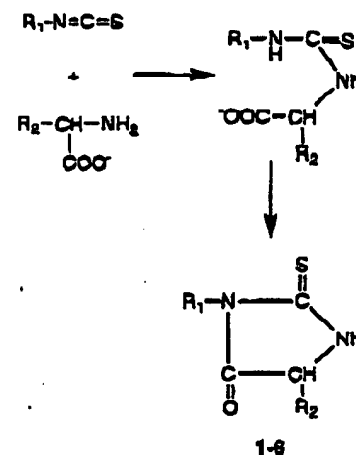

Figure 1. Formation of 3,5-disubstituted 2-thiohydantoins (1–6). **1:** R$_1$ = allyl; R$_2$ = isopropyl. **2:** R$_1$ = allyl; R$_2$ = benzyl. **3:** R$_1$ = allyl; R$_2$ = indolylmethyl. **4:** R$_1$ = 4-(methylthio)-3-butenyl; R$_2$ = isobutyl. **5:** R$_1$ = 4-(methylthio)-3-butenyl; R$_2$ = benzyl. **6:** R$_1$ = 4-(methylthio)-3-butenyl; R$_2$ = 2-(methylthio)ethyl.

sulfate, was evaporated under reduced pressure to give a solid residue, which was then recrystallized in EtOH. The crystal products were analyzed by UV, IR, EI-MS, and NMR spectroscopies to confirm their structures (Figure 1). The spectral data obtained were as follows.

3-Allyl-5-isopropyl-2-thiohydantoin (1): UV λ_{max} (EtOH) 267 nm; HR-EI-MS *m/z* (M$^+$) calcd for C$_9$H$_{14}$ON$_2$S 198.0796, found 198.0811; LR-EI-MS *m/z* (%, rel intens.) 198 (M$^+$, 100), 183 (18), 170 (37), 155 (27), 99 (49), 72 (97), 55(40), 41 (54); IR ν_{max} (KBr) cm^{-1} 3295 (NH), 1725 (C=O), 1508 (N–C=S), 1171 (C=S); ^{1}H NMR ((CD$_3$)$_2$CO) δ (ppm) 9.13 (1H, s), 5.51–5.59 (1H, m), 4.76–4.95 (2H, m), 4.18–4.31 (2H, m), 4.17–4.18 (1H, m), 2.19–2.28 (1H, m), 1.08 (3H, d, *J* = 6.8 Hz), 0.92 (3H, d, *J* = 6.8 Hz); ^{13}C NMR ((CD$_3$)$_2$CO) δ (ppm) 184.7, 174.3, 132.6, 117.7, 64.9, 48.2, 31.6, 18.6, 16.6.

3-Allyl-5-benzyl-2-thiohydantoin (2): UV λ_{max} (EtOH) 269 nm; HR-EI-MS *m/z* (M$^+$) calcd for C$_{13}$H$_{14}$ON$_2$S 246.0827, found 246.0823; LR-EI-MS *m/z* (%, rel intens.) 246 (M$^+$, 99), 155 (15), 91 (100), 77 (13), 65 (30), 51(11); IR ν_{max} (KBr) cm^{-1} 3205 (NH), 1747 (C=O), 1521 (N–C=S), 1174 (C=S); ^{1}H NMR ((CD$_3$)$_2$CO) δ (ppm) 9.16 (1H, s), 7.20–7.28 (5H, m), 5.51–5.59 (1H, m), 4.76–4.95 (2H, m), 4.59 (1H, dd, *J* = 4.9 & 5.6 Hz), 4.18–4.21 (2H, m), 3.23 (1H, dd, *J* = 4.9 & 14.2 Hz), 3.10 (1H, dd, *J* = 5.9 & 14.2 Hz); ^{13}C NMR ((CD$_3$)$_2$CO) δ (ppm) 184.2, 174.1, 135.9, 132.2, 130.7, 129.2, 127.9, 117.1, 60.8, 43.1, 37.3.

3-Allyl-5-(indolylmethyl)-2-thiohydantoin (3): UV λ_{max} (EtOH) 268 nm; LR-EI-MS *m/z* (%, rel intens.) 285 (M$^+$, 15), 130 (100); IR ν_{max} (KBr) cm^{-1} 3413 (NH), 1732 (C=O), 1533 (N–C=S), 1174 (C=S); ^{1}H NMR ((CD$_3$)$_2$CO) δ (ppm) 10.10 (1H, s), 9.13 (1H, s), 7.60 (1H, d, *J* = 7.8 Hz), 7.36 (1H, d, *J* = 7.8 Hz), 7.19 (1H, d, *J* = 2.4 Hz), 7.08 (1H, t, *J* = 6.8 Hz), 7.01 (1H, t, *J* = 6.8 Hz), 5.46–5.55 (1H, m), 4.75–4.80 (2H, m), 4.59 (1H, dd, *J* = 4.9 & 5.6 Hz), 4.18–4.19 (2H, m), 3.37 (1H, dd, *J* = 6.4 & 14.6 Hz), 3.27 (1H, dd, *J* = 4.9 & 14.7 Hz); ^{13}C NMR ((CD$_3$)$_2$CO) δ (ppm) 184.3, 174.6, 137.4, 132.2, 128.5, 125.0, 122.2, 119.7, 119.4, 117.0, 112.1, 108.9, 60.7, 43.1, 27.5.

3-[4-(Methylthio)-3-butenyl]-5-isobutyl-2-thiohydantoin (4): UV λ_{max} (EtOH) 267 nm; HR-EI-MS *m/z* (M$^+$) calcd for C$_{12}$H$_{20}$ON$_2$S$_2$ 272.1017, found 272.0969; LR-EI-MS *m/z* (%, rel intens.) 272 (M$^+$, 19), 225 (57), 100 (91), 85 (100); IR ν_{max} (KBr) cm^{-1} 3166 (NH), 1749 (C=O), 1527 (N–C=S), 1163 (C=S); ^{1}H NMR ((CD$_3$)$_2$CO) δ (ppm) 9.11 (1H, s), 6.08 (1H, d, *J* = 15.1 Hz), 5.33 (1H, dt, *J* = 7.3 & 14.7 Hz), 4.21–4.25 (1H, m), 3.79 (2H, t, *J* = 7.3 Hz), 2.42–2.52 (2H, m), 2.21 (3H, s), 1.89–1.98 (1H, m), 1.57–1.70 (2H, m), 0.97 (3H, d, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.4 Hz); ^{13}C NMR ((CD$_3$)$_2$CO) δ (ppm) 184.4, 175.7, 127.4, 122.5, 58.3, 41.4, 40.8, 32.1, 25.2, 23.4, 21.9, 14.4.

3-[4-(Methylthio)-3-butenyl]-5-benzyl-2-thiohydantoin (5): UV λ_{max} (EtOH) 269 nm; HR-EI-MS *m/z* (M$^+$) calcd

for C₁₅H₁₈ON₂S₂ 306.0861, found 306.0859; LR-EI-MS m/z (%, rel intens.) 306 (M⁺, 6), 259 (27), 100 (43), 91 (15), 85 (100), 77 (4), 65 (4), 51 (2); IR νmax (KBr) cm⁻¹ 3203 (NH), 1747 (C=O), 1519 (N−C=S), 1162 (C=S); ¹H NMR (CDCl₃) δ (ppm) 7.70 (1H, s), 7.32 (1H, t, J = 6.8 Hz), 7.27 (1H, t, J = 7.3 Hz), 7.20 (1H, d, J = 6.8 Hz). 6.02 (1H, d, J = 15.1 Hz), 5.26 (1H, dt, J = 7.3 & 14.6 Hz), 4.30 (1H, dd, J = 3.9 & 8.8 Hz), 3.77 (2H, t, J = 7.3 Hz), 3.28 (1H, dd, J = 3.9 & 14.2 Hz), 2.87 (1H, dd, J = 8.8 & 14.2 Hz), 2.30−2.42 (2H, m), 2.21 (3H, s); ¹³C NMR (CDCl₃) δ (ppm) 183.6, 179.4, 134.7, 129.2, 129.2, 129.0, 127.6, 126.9, 121.2, 60.4, 40.5, 37.6, 31.2, 14.2.

3-[4-(Methylthio)-3-butenyl]-5-[2-(methylthio)ethyl]-2-thiohydantoin (6): UV λmax (EtOH) 268, 228 nm; IR νmax (EtOH) cm⁻¹ 3567 (NH), 1740 (C=O), 1541 (N−C=S), 1157 (C=S); LR-EI-MS m/z (rel intens. %) 290 (M⁺, 2.6), 243 (15.8), 100 (34.2), 85 (100), 72 (3.8), 61 (19.1); ¹H NMR ((CD₃)₂CO) δ (ppm) 9.12 (1H, s), 6.09 (1H, d, J = 15.1 Hz), 5.35 (1H, dt, J = 7.3 & 14.7 Hz), 4.35−4.38 (1H, m), 3.81 (2H, t, J = 6.8 Hz), 2.66 (2H, t, J = 7.3 Hz), 2.43−2.53 (2H, m), 2.21 (3H, s), 2.11−2.19 (1H, m), 2.09 (3H, s), 1.97−2.03 (1H, m); ¹³C NMR ((CD₃)₂CO) δ (ppm) 184.6, 175.2, 127.4, 122.8, 58.6, 40.9, 32.1, 31.5, 29.6, 14.9, 14.4.

Measurement of pH Effect. A mixture of 100 μmol of AITC and 500 μmol of Val or Phe in 10 mL of 0.1 M MacIlvaine buffer (pH 3.0−9.0) was stirred for 48 h at 36 °C. The reaction mixture was extracted with ethyl acetate, and the extract was then analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu LC-5A liquid chromatograph with a UV detector and a LiChrosorb RP-18 column (250-mm × 4-mm i.d.; Merck, Tokyo, Japan). The mobile phase used was a mixture of methanol/water (70/30, v/v) and was run at 0.8 mL/min. Detection was made at 270 nm. Amounts of the 2-thiohydantoins formed were measured by comparing their peak areas with those of the authentic specimens.

Antimicrobial Activity Assay. The paper disk diffusion method (Heisey and Gorham, 1992) was used with *Aspergillus fumigatus* (IFO 4311), *Eurotium chevalieri* (IFO 4090), *Penicillium expansum* (IFO 8800), *Candida albicans* (IFO 1061), *Schizosaccharomyces pombe* (IFO 0638), *Zygosaccharomyces rouxii* (IFO 1058), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311), *Salmonella typhimurium* TA 98, and *Staphylococcus aureus* (ATCC 12598). The fungi and the bacteria were cultured, respectively, on a glucose−peptone agar (pH 5.0; Nissui Co., Tokyo Japan) and a soybean−casein digest broth (pH 7.0). A 10-mm diameter paper disk, to which was added 25 μL of a dimethyl sulfoxide (DMSO) solution containing 0, 300, 600, or 1000 μg of the 2-thiohydantoins, was put on the center of an agar plate (90 mm i.d.) seeded with the fungi ((4−5) × 10⁶ colony forming unit (CFU)) or the bacteria ((2−3) × 10⁸ CFU) and then incubated for 72−96 h at 25 °C for the fungi and for 48 h at 37 °C for the bacteria. The growth-inhibition zone was measured. The antimicrobial assay was also carried out using the bacterial strains in 2.0 mL of a soybean−casein digest broth (pH 7.0), in which the bacteria were similarly incubated with the same amounts of the test compounds as in agar plates, and their growth was measured photometrically at 660 nm. All assays were done in triplicate. Antimicrobial activity is expressed as the difference in the growth-inhibition zone and/or the optical density at 660 nm recorded with or without the test compounds.

Antimutagenicity Assay. The modification (Nohmi et al., 1986) of the procedure of Maron and Ames (1983) was used with *S. typhimurium* TA 98 as the bacterial strain and IQ as the mutagen. A mixture of 0.1 mL of the test compound (0−500 μg) in DMSO, 0.1 mL of IQ (0 or 0.5 μg) in DMSO, 0.3 mL of the overnight-cultured bacterial suspension (2 × 10⁹ CFU), and 0.5 mL of S9 mix (10% S9) was incubated for 20 min at 37 °C. The bacteria were twice rinsed with 0.1 M phosphate buffer (pH 7.4) to remove the test compound and the mutagen and were then spread on a plate with 2 mL of top agar. After a 48-h culture at 37 °C, the revertants that resulted were counted. In another experiment, the test compounds were added to the bacterial suspension which had been incubated with the same amount of IQ and S9 mix for 20 min at 37 °C,
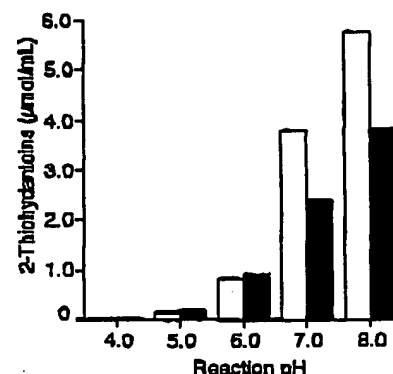

Figure 2. Effect of pH on the formation of compounds 1 (□) and 2 (■).

and then they were further incubated for 20 min. Thereafter the same procedure as described above was followed. Each sample was assayed using duplicate plates. Spontaneous revertants were subtracted from the number of revertants in all plates exposed to IQ in the presence or absence of the test compounds, and those found in the plates exposed to IQ in the absence of the test compounds are defined as 100% mutagenicity (positive control). The data are presented as the mean ± standard deviation of three independent assays. Significant difference comparison with the positive control is based on *p* value differences among the means. Simple regression analyses of the data were run to determine the relationship between the antimutagenic activity of the 3,5-disubstituted 2-thiohydantoins and their concentrations using the SAS General Linear Models Procedure (SAS, 1987).

RESULTS AND DISCUSSION

Effect of pH on the Formation of the 3,5-Disubstituted 2-Thiohydantoins. To study the effect of pH on the formation of the 3,5-disubstituted 2-thiohydantoins, a model reaction was carried out using AITC, Val, and Phe. The results are shown in Figure 2. The 2-thiohydantoins 1 and 2 formed best at alkaline pH's. Our previous study (Matsuoka et al., 1997) showed that MTBI was converted into dithiocarbamates by reaction with methanethiol at pH's over 6.0. The present study showed that both compounds 1 and 2 could be formed at neutral and weakly acidic pH's (5.0−6.0), suggesting that a certain amount of 2-thiohydantoin derivatives can be formed in isothiocyanate-containing foods or seasonings, and even in the human digestive tract. This finding needs to be further confirmed, as does the time required for the 2-thiohydantoin derivatives' formation.

Effect of the 3,5-Disubstituted 2-Thiohydantoins on the Growth of Microorganisms. Froelich et al. (1954) reported that synthetic 5-alkyl-2-thiohydantoins had a growth-inhibitory effect on tubercle bacilli. Marton et al. (1993) showed that some 5-(arylmethylene)-2-thiohydantoins were fungicidal and bactericidal against plant pathogens but that no biological activity occurred with their 5-(alkylmethyl) analogues. Their results also suggested that the antimicrobial activity depends on the structure of the substituted groups of 2-thiohydantoins. In the present study, the six 3,5-disubstituted 2-thiohydantoins (1−6) were examined for their effect on the growth of 10 strains of microorganisms, including 4 bacteria, 3 yeasts, and 3 molds. This experiment also aimed to confirm whether the compounds have any disturbing effect on the antimutagenicity assay with *S. typhimurium* TA 98. All compounds examined showed no antimicrobial activity at a dose range from 300 to
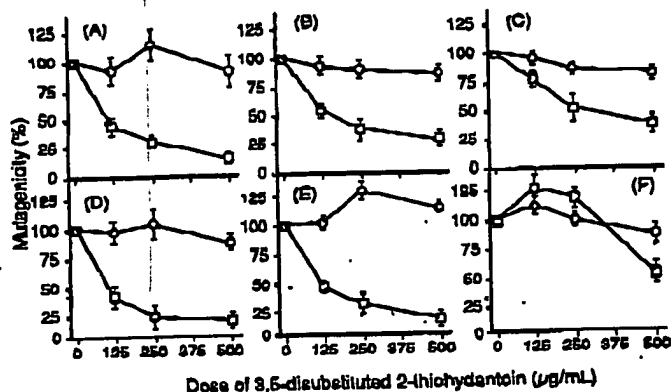
Takahashi et al.

Figure 3. Inhibitory effects of the 3,5-disubstituted 2-thiohydantoins on the mutagenicity of IQ: (A) compound 1; (B) compound 2; (C) compound 3; (D) compound 4; (E) compound 5; (F) compound 6. Symbols: O, the bacterial strain that had been incubated with IQ (0.5 μg/mL) and S9 mix for 20 min at 37 °C was further incubated with the six test compounds for 20 min; □, the bacterial strain was simultaneously incubated with the test compounds for 20 min at 37 °C in the presence of IQ (0.5 μg/mL) and S9 mix. The mutagenicity is expressed in terms of the percentage in the revertant population found in the presence or absence of the test compounds, where the mutagenicity of IQ is defined as 100%. The number of revertants found spontaneously was 26 ± 7.

1000 μg/plate toward any of the microorganisms including *S. typhimurium* TA 98 (data not shown). In a soybean–casein digest broth, the same results were observed against the four bacterial strains (data not shown). The results clearly demonstrated that these 3,5-disubstituted 2-thiohydantoins had no antimicrobial activity at dose levels below 1000 μg/plate and, thus, suggested that no cytotoxic effect of the 3,5-disubstituted 2-thiohydantoins on *S. typhimurium* TA 98 in the antimutagenicity assay occurs at the dose levels. Minimum inhibitory concentrations of the test compounds were not determined, because solubility of the compounds was marginal at a concentration of 1000 μg/25 μL in DMSO.

Antimutagenic Property of the 3,5-Disubstituted 2-Thiohydantoins. The six 3,5-disubstituted 2-thiohydantoins were assayed for their mutagenic and antimutagenic activity. None of the compounds examined had mutagenic activity on *S. typhimurium* TA 98 with or without S9 mix (data not shown). As shown in Figure 3, however, when the bacterial strain and the test compounds (125–500 μg/mL) were simultaneously treated with IQ (0.5 μg/mL) in the presence of S9 mix, the compounds except for 6 showed a dose-dependent inhibition of the mutagenicity of IQ. No toxicity on *S. typhimurium* TA 98 was microscopically observed in all compounds at a 125–500 μg/mL dose in the presence or absence of IQ. In this dose range, percentage of suppressing mutagenicity of IQ and coefficient of determination (r^2) from the simple regressions analyses were for compound 1, 56–86% ($r^2 = 0.94$; $p < 0.001$); for compound 2, 45–74% ($r^2 = 0.90$; $p < 0.005$); for compound 3, 23–63% ($r^2 = 0.81$; $p < 0.025$); for compound 4, 59–81% ($r^2 = 0.71$; $p < 0.01$); and for compound 5, 54–83% ($r^2 = 0.91$; $p < 0.005$). Compound 6 showed no inhibition of the IQ mutagenicity up to 250 μg/mL but was antimutagenic (46% inhibition; $p < 0.005$) at 500 μg/mL. Compound 4 was the most suppressive against the mutagenicity of IQ at 125 μg/mL (59% inhibition; $p < 0.001$) and was followed by both compounds 1 (56% inhibition; $p < 0.001$) and 5 (54%

inhibition; $p < 0.005$) at the same dose level. At 500 μg/mL, these three compounds were nearly equal in their antimutagenic activity. The six 3,5-disubstituted 2-thiohydantoins examined appeared to be $4 \geq 1 \geq 5 > 2 > 3 > 6$ in order of their antimutagenic effects. In contrast, when the bacterial strain, which was pretreated with IQ in the presence of S9 mix for 20 min, was further incubated for 20 min with the test compounds, disappearance of the antimutagenic effect was observed in compounds 1, 4, and 5, and a large decrease in the inhibition was shown with both compounds 2 (5–16% inhibition; $r^2 = 0.63$; $p < 0.05$) and 3 (5–19% inhibition; $r^2 = 0.66$; $p < 0.05$). Compound 6 showed a weak inhibition (14%; $p < 0.05$) of the mutagenicity of IQ at only a 500 μg/mL dose. In these experiments, the mean number of revertants found in the IQ-treated plates with S9 mix in the absence of the test compounds was 826/plate, indicating that IQ was activated by the S9 mix-mediated metabolic system. These results demonstrate that all compounds could inhibit the S9 mix-mediated activation of IQ when incubated simultaneously with IQ, though compound 6 needed a higher concentration (500 μg/mL) for the inhibition. S9 fraction from rat liver contains heterocyclic amine (HCA)-metabolizable enzymes P-450 1A1 and 1A2 (Kamataki et al., 1983; Soucek and Gut, 1992). The enzymes metabolize HCA including IQ to N-hydroxy-HCA, which is subsequently converted into an ultimate form being highly active on DNA (Ishii et al., 1980; Hamilton and Teel, 1996; Yamazoe et al., 1983). Therefore, the antimutagenic activity of the 3,5-disubstituted 2-thiohydantoins might be attributed to the inhibition of the cytochromes P-450.

Although there has been no available study on the mutagenicity and/or antimutagenicity of 3,5-disubstituted 2-thiohydantoins derived from naturally occurring isothiocyanates and amino acids, such derivatives of 2-thiohydantoins as 5-[(5-bromo-2-thienyl)methylene]-3-(morpholinomethyl)-2-[(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)thio]hydantoin and 5-[(5-bromo-2-thienyl)-methylene]-2-thiohydantoin were shown to act as antitumor agents against human cell lines including leukemia cell lines (Al-Obaid et al., 1996). Their related hydantoin, 5,5-bis(4-chlorophenyl)-1,3-dichlorohydantoin, similarly showed a suppressive effect on P-388 lymphocytic leukemia in mice (Rodgers et al., 1977). Modes of antitumorigenicity of these 2-thiohydantoins and the hydantoin were not shown. These literature references suggest that some 2-thiohydantoins are able to behave as anticarcinogenic compounds depending on their substituents at the 3 and/or 5 positions. From the results in Figure 3, it appears that the antimutagenic activity of the 3,5-disubstituted 2-thiohydantoins examined depends on the substituents at position 5 rather than at position 3.

As for MTBI used in this study, there has been no available information about mutagenicity and/or antimutagenicity. AITC, another isothiocyanate used for the preparation of the 3,5-disubstituted 2-thiohydantoins, was mutagenic on *S. typhimurium* TA 100 with a rat liver homogenate having an activity to convert AITC to an epoxide intermediate (Neudecker and Henschler, 1985), but its antimutagenic property against HCA is unknown. Recently, Musk et al. (1995) reported that AITC was unable to induce either chromosome aberrations or sister chromatid exchanges even at highly cytotoxic doses. Thus, it is still unclear whether

MTBI and AITC changed in their behavior against IQ by their conversion into the corresponding 2-thiohydantoins.

Figure 3 demonstrates that the 3,5-disubstituted 2-thiohydantoins were able to inhibit the IQ mutagenicity at concentrations of either 125–500 or 500 μg/mL when incubated simultaneously with IQ in the presence of S9 mix. Kada and Shimoi (1987) classified inhibitors of mutagenesis into desmutagens and bio-antimutagens, of which the former acts to inactivate mutagens by such modes of action as structural modulation, absorption, or inhibition of metabolic activation of the mutagens and the latter acts on a stage of the repair of DNA lesions caused by mutagens. Wattenberg (1993) divided inhibitors of carcinogenesis into three categories, in which factors that can act effectively by such means as inhibition of metabolic activation of genotoxic compounds and trapping of DNA-reactive compounds when administered prior to or simultaneously with genotoxic compounds were characterized as blocking agents. According to their definition, the 3,5-disubstituted 2-thiohydantoins can act as a desmutagen and as a blocking agent. Of the six 3,5-disubstituted 2-thiohydantoins examined, compounds 2 and 3 showed a lower antimutagenicity even when added after metabolic activation of IQ with S9 mix. This suggested that both compounds may have a weak activity to inhibit the S9-mediated metabolic activation of IQ and a reactivity with its activated intermediate(s). Further investigations are needed to elucidate in detail the antimutagenic mechanisms of the 3,5-disubstituted 2-thiohydantoins along with their structure–activity relationships.

ABBREVIATIONS USED

AITC, allyl isothiocyanate; CFU, colony forming unit; HR-EI-MS, high-resolution electron impact mass spectrometry; HCA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; IR, infrared spectrometry; Leu, L-leucine; LR-EI-MS, low-resolution electron impact mass spectrometry; Met, L-methionine; MTBI, 4-(methylthio)-3-butenyl isothiocyanate; NMR, nuclear magnetic resonance spectrometry; PEITC, 2-phenylethyl isothiocyanate; Phe, L-phenylalanine; S9 mix, a mixture of rat microsomal fraction and cofactors; Trp, L-tryptophan; UV, ultraviolet spectrometry; Val, L-valine; 1, 3-allyl-5-isopropyl-2-thiohydantoin; 2, 3-allyl-5-benzyl-2-thiohydantoin; 3, 3-allyl-5-(indolylmethyl)-2-thiohydantoin; 4, 3-[4-(methylthio)-3-butenyl]-5-isobutyl-2-thiohydantoin; 5, 3-[4-(methylthio)-3-butenyl]-5-benzyl-2-thiohydantoin; 6, 3-[4-(methylthio)-3-butenyl]-5-[2-(methylthio)ethyl]-2-thiohydantoin.

LITERATURE CITED

Abu-Shakra, A.; Ioannides, C.; Walker, R. Metabolic activation of 2-amino-3-methylimidazo(4,5-f)quinoline by hepatic preparations-contribution of the cytosolic fraction and its significance to strain differences. Mutagenesis 1986, 1, 367–370.

Al-Obaid, A. M.; El-Subbagh, H. I.; Khodair, A. I.; Elmazar, M. M. A 5-Substituted-2-thiohydantoin analogues as a novel class of antitumor agents. Anti-Cancer Drugs 1996, 7, 873–880.

Ashworth, M. R. F. The Determination of Sulphur-containing Groups; Academic Press: New York, 1972; Vol. 1, pp 90–115.

Barcelo, S.; Gardiner, J. M.; Gescher, A.; Chipman, J. K. CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. Carcinogenesis 1996, 17, 277–282.

Chen, C.-W.; Ho, C.-T. Thermal degradation of allyl isothiocyanate in aqueous solution. J. Agric. Food Chem. 1998, 46, 220–223.

Cortes, S.; Liao, Z.-K.; Watson, D.; Kohn, H. Effect of structural modification of the hydantoin ring on anticonvulsant activity. J. Med. Chem. 1985, 28, 601–606.

Drobnica, L.; Augustin, J. Reaction of isothiocyanates with amino acids, peptides and proteins. I. Kinetics of the reaction of aromatic isothiocyanates with glycine. Collect. Czech. Chem. Commun. 1965a, 30, 99–104.

Drobnica, L.; Augustin, J. Reaction of isothiocyanates with amino acids, peptides and proteins. II. Kinetics of the reaction of aromatic isothiocyanates with amino acids and cyclization of the addition products. Collect. Czech. Chem. Commun. 1965b, 30, 1221–1228.

Edman, P. Method for determination of the amino acid sequence in peptides. Acta Chem. Scand. 1950, 4, 283–293.

El-Barbary, A. A.; Khodair, A. I.; Pedersen, E. B.; Nielsen, C. S-Glucosylated hydantoins as new antiviral agents. J. Med. Chem. 1994, 37, 73–77.

Froelich, E.; Fruehan, A.; Jackman, M.; Kirchner, F. K.; Alexander, E. J.; Archer, S. 5-Heptyl-2-thiohydantoin, a new antitubercular agent. J. Am. Chem. Soc. 1954, 76, 3099–3100.

Hamilton, S. M.; Teel, R. W. Effects of isothiocyanates on cytochrome P-450 1A1 and 1A2 activity and on the mutagenicity of heterocyclic amines. Anticancer Res. 1996, 16, 3597–3602.

Hamilton, S. M.; Zhang, Z.; Teel, R. W. Effects of isothiocyanate alkyl chain-length on hamster liver cytochrome P-450 activity. Cancer Lett. 1994, 82, 217–224.

Hatch, F. T.; Felton, J. S.; Stuermer, D. H.; Bjeldanes, L. F. Identification of mutagens from the cooking of food. In Chemical Mutagens: Principles and Methods of their Detection; De Serres, F. J., Ed.; Plenum Press: New York, 1984; pp 111–164.

Heisey, R. M.; Gorham, B. K. Antimicrobial effects of plant extracts on Streptococcus mutans, Candida albicans, Trichophyton rubrum and other microorganisms. Lett. Appl. Microbiol. 1992, 14, 136–139.

Ishii, K.; Yamazoe, Y.; Kamataki, T.; Kato, R. Metabolic activation of mutagenic tryptophan pyrolysis products by rat liver microsomes. Cancer Res. 1980, 40, 2596–2600.

Jägerstad, M.; Laser-Reuterswärd, A.; Öste, R.; Dahlqvist, A.; Grivas, S.; Olsson, K.; Nyhammar, T. Creatinine and Maillard reaction products as precursors of mutagenic compounds formed in fried beef. In The Maillard Reaction in Foods and Nutrition; Waller, G. R., Feather, M. S., Eds.; ACS Symposium Series 215: American Chemical Society: Washington, DC, 1983; pp 507–519.

Kada, T.; Shimoi, K. Desmutagens and bio-antimutagens: their modes of action. Bioessays 1987, 7, 113–116.

Kamataki, T.; Maeda, K.; Yamazoe, Y.; Matsuda, N.; Ishii, K.; Kato, R. A high spin form of cytochrome P-450 highly purified from PCB-treated rats: catalytic characterization and immunological quantitation in liver microsomes. Mol. Pharmacol. 1983, 24, 146–155.

Kammueller, M. E.; Seinen, W. Structural requirements for hydantoins and 2-thiohydantoins to induce lymphoproliferative popliteal lymph node reactions in the mouse. Int. J. Immunopharmacol. 1988, 10, 997–1010.

Kasai, H.; Yamaizumi, Z.; Wakabayashi, K.; Nagao, M.; Sugimura, T.; Yokoyama, S.; Miyazawa, T.; Spingarn, N. E.; Weisburger, J. H.; Nishimura, S. Potent novel mutagens produced by broiling fish under normal conditions. Proc. Jpn. Acad. 1980, B56, 278–283.

Kawakishi, S.; Namiki, M. Decomposition of allyl isothiocyanate in aqueous solution. Agric. Biol. Chem. 1969, 33, 452–459.

Kawakishi, S.; Namiki, M.; Watanabe, H.; Muramatsu, K. The decomposition products of sinalbin and their degradation pathways. Agric. Biol. Chem. 1967, 31, 823–30.

Knasmuller, S.; Friesen, M. D.; Holme, J. A.; Alexander, J.; Sanyal, R.; Kassie, F.; Bartsch, H. Effects of phenethyl isothiocyanate on metabolism and on genotoxicity of di-

Takahashi et al.

methylnitrosamine and 2-amino-1-methyl-6-phenylimidazo-[4,5-beta]pyridine (PhIP). *Mutat. Res.* 1996, *350*, 93–102.

Kosemura, S.; Yamamura, S.; Hasegawa, K. Chemical studies on 4-methylthio-3-butenyl isothiocyanate from roots of Japanese radish (*Raphanus sativus* L.) in connection with raphanusanins, phototropism-regulating substances of radish hypocotyls. *Tetrahedron Lett.* 1993, *34*, 481–484.

Marinchev, A.; Mirtcheva, J.; Sidjimov, A.; Himova, M. Induction of lymphoproliferative popliteal lymph node reaction by hydantoin derivatives: structure–activity relationships. *Int. J. Immunopharmacol.* 1995, *17*, 981–94.

Maron, D. M.; Ames, B. N. Revised methods for *Salmonella* mutagenicity test. *Mutat. Res.* 1983, *113*, 173–215.

Marton, J.; Enisz, J.; Hosztafi, S.; Timár, T. Preparation and fungicidal activity of 5-substituted hydantoins and their 2-thio analogues. *J. Agric. Food Chem.* 1993, *41*, 148–152.

Matsuoka, H.; Toda, Y.; Yanagi, K.; Takahashi, A.; Yoneyama, K.; Uda, Y. Formation of thioxopyrrolidines and dithiocarbamates from 4-methylthio-3-butenyl isothiocyanates, the pungent principle of radish, in aqueous media. *Biosci. Biotechnol. Biochem.* 1997, *61*, 2109–2112.

Morse, M. A.; Amin, S. G.; Hecht, S. S.; Chung, F.-L. Effects of aromatic isothiocyanates on tumorigenicity. O6-methyl-guanine formation and metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse lung. *Cancer Res.* 1989, *49*, 2894–2897.

Morse, M. A.; Eklind, K. I.; Hecht, S. S.; Jordan, K. G.; Chok, O.-I.; Desai, D. H.; Amin, S. G.; Chung, F.-L. Structure–activity relationship for inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone lung tumorigenesis by arylalkyl isothiocyanates in A/J mice. *Cancer Res.* 1991, *51*, 1846–1850.

Musk, S. R.; Smith, T. K.; Johnson, I. T. On the cytotoxicity and genotoxicity of allyl and phenethyl isothiocyanates and their parent glucosinolates sinigrin and gluconasturtiin. *Mutat. Res.* 1995, *348*, 19–23.

Neudecker, T.; Henschler, D. Allyl isothiocyanate is mutagenic in *Salmonella typhimurium*. *Mutat. Res.* 1985, *156*, 33–37.

Nohmi, T.; Matsui, M.; Ishidate, M., Jr. Method for the induced-mutation frequency test using bacterial tester strains. *Toxicol. Forum* 1986, *9*, 189–198.

Ohgaki, H.; Kusama, K.; Matsukura, N.; Morino, K.; Hasegawa, H.; Sato, S.; Takayama, S.; Sugimura, T. Carcinogenicity in mice of a mutagenic compound 2-amino-3-methylimidazo(4,5-f)quinoline, from broiled sardine, cooked beef and beef extract. *Carcinogenesis* 1984, *5*, 921–924.

Ohgaki, H.; Takayama, S.; Sugimura, T. Carcinogenicities of heterocyclic amines in cooked food. *Mutat. Res.* 1991, *259*, 399–410.

Rodgers, T. R.; LaMontagne, M. P.; Markovac, A.; Ash, A. B. Hydantoins as antitumor agents. *J. Med. Chem.* 1977, *20*, 591–594.

SAS Institute Inc. *SAS/STAT Guide for Personal Computers*, version 6; SAS Institute: Cary, NC, 1987.

Soucek, P.; Gut, I. Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica* 1992, *22*, 83–103.

Spingarn, N. E.; Kasai, H.; Vuolo, L. L.; Nishimura, S.; Yamaizumi, Z.; Sugimura, T.; Matsushima, T.; Weisburger, J. H. Formation of mutagens in cooked foods. III. Isolation of a potent mutagen from beef. *Cancer Lett.* 1980, *9*, 177–183.

Sugimura, T.; Nagao, M.; Kawachi, T.; Honda, M.; Yahagi, T.; Seino, Y.; Sato, S.; Matsukura, N.; Matsushita, T.; Shirai, A.; Sawamura, M.; Matsumoto, H. Mutagen-carcinogens in foods with special reference to highly mutagenic pyrolytic products in broiled food. In *Origins of Human Cancer*; Hiatt, H. H., Watson, J. D., Winsten, J. A., Eds.; Cold Spring Harbor Laboratory Press: New York, 1977; pp 1561–1577.

Takayama, S.; Nakatsuru, Y.; Masuda, M.; Ohgaki, H.; Sato, S.; Sugimura, T. Demonstration of carcinogenicity in F344 rats of 2-amino-3-methylimidazo(4,5-f)quinoline from broiled sardine, fried beef and beef extract. *Gann* 1984, *75*, 467–470.

Uda, Y.; Ozawa, Y.; Ohshima, T.; Kawakishi, S. Identification of enolated 2-thioxo-3-pyrrolidinecarbaldehyde, a new degradation product of 4-methylthio-3-butenyl isothiocyanate. *Agric. Biol. Chem.* 1990, *54*, 613–617.

VanEtten, C. H.; Wolff, I. A. Natural sulfur compounds. In *Toxicants Occurring Naturally in Foods*; Committee on Food Protection, Food and Nutrition Board, National Research Council, Eds.; National Academy of Sciences: Washington, DC, 1973; pp 210–234.

Wakabayashi, K.; Nagao, M.; Ochiai, M.; Tahira, T.; Yamaizumi, Z.; Sugimura, T. A mutagen precursor in Chinese cabbage, indole-3-acetonitrile, which becomes mutagenic on nitrite treatment. *Mutat. Res.* 1985, *143*, 17–21.

Wattenberg, L. W. Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanate and related compounds. *J. Natl. Cancer Inst.* 1977, *58*, 395–398.

Wattenberg, L. W. Inhibitory effects of benzyl isothiocyanate administered shortly before diethylnitrosamine or benzo[a]pyrene on pulmonary and forestomach neoplasia in A/J mice. *Carcinogenesis* 1987, *8*, 1971–1973.

Wattenberg, L. W. Inhibition of carcinogenesis by nonnutrient constituents of the diet. In *Food and Cancer Prevention: Chemical and Biological Aspects*; Waldron, K. W., Johnson, I. T., Fenwick, G. R., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1993; pp 12–23.

Wattenberg, L. W.; Loub, W. D. Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res.* 1978, *38*, 1410–1413.

Yamazoe, U.; Shimada, M.; Kamataki, T.; Kato, R. Microsomal activation of 2-amino-3-methylimidazo(4,5-f)quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Res.* 1983, *43*, 5768–5744.

Zhang, Y.; Talalay, P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res.* 1994, *54* (Suppl.), 1976s–1981s.